Acid-Sensing Ion Channels in Oxygen-Glucose Deprivation

Undergraduate Research Thesis

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By

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Abstract

Acid-sensing ion channels (ASICs) are activated by extracellular pH fluctuations to play critical roles in normal brain functions. They also contribute to brain damage following ischemic stroke when blood flow is cut off from parts of the brain causing the extracellular pH to drop. Inhibiting ASIC1a in animal models of ischemia prevents much of this permanent brain damage, implicating ASIC1a as a novel therapeutic target. Surprisingly, little is known about the mechanism of ASIC-mediated cell death, and there are currently no drugs to specifically inhibit ASIC1a in patients. The Askwith lab has found that activation of the delta opioid receptor (DOR) in neuronal cultures attenuates ASIC-mediated cell death. Further, the prominent hypothesis on the mechanism of DOR-induced neuroprotection involves interference with the association between ASIC1a and the Receptor Interacting Protein Kinase 1 (RIP1) and RIP1 phosphorylation. The purpose of this study is to optimize a method to quantify ASIC-mediated cell death in hippocampal slices subjected to oxygen-glucose deprivation (OGD) in order to mimic cerebral ischemia more accurately than in cell culture and test the molecular hypothesis on this novel mechanism of neuroprotection. In order to attain this goal, we treated mouse acute hippocampal slices with either OGD or control conditions then stained cells to quantify death. Results showed increased cell death following OGD treatment relative to the control. Intervention of this model with DOR activation via SNC80 during the OGD period showed attenuation of ASIC-mediated cell death, implicating DOR activation as a novel target for the regulation of this cell death pathway. Cell death was also reduced by inhibition of RIP1 via necrostatin-1, suggesting that the mechanism of this cell death is dependent on RIP1 activity. These data indicate that our model replicates observations from neuronal culture and that this system is a useful model to study ASIC-mediated cell death.

Chapter 1: Introduction

Acid-sensing ion channels (ASICs) function as proton-gated channels and post-synaptic receptors that are essential for normal brain functions including fear, synaptic plasticity, memory (Du et al., 2014; Wemmie et al., 2003), and retinal function (Ettaiche et al., 2006). In addition to their involvement in normal physiological activity, they are also key players in a variety of other situations involving pH fluctuations including anxiety (Pidoplichko et al., 2014), addictive behaviors (Kreple et al., 2014), depression (Coryell et al., 2009), seizure termination (Ziemann et al., 2008), and pain (Bohlen et al., 2011; Diochot et al., 2012). A critical role of acid-sensing ion channels more closely examined in this thesis is their contribution to neuronal death and brain damage following cerebral acidosis as in ischemic stroke (Chu et al., 2013; Siesjo, 1988; Xiong et al., 2004, 2008; Yang et al., 2011). During an ischemic stroke, blood flow is cut off from parts of the brain causing the pH to drop and activating ASIC1a (Chu et al., 2013; Kagansky et al., 2001; Xiong et al., 2004, 2008; Yang et al., 2011). When ASIC1a is inhibited following an ischemic event in animal models, much of the cell death is prevented (Xiong et al., 2004; Yang et al., 2011). Thus, ASIC1a represents a potential therapeutic target to prevent permanent brain damage following stroke. My thesis addresses ASIC-dependent cell death and the development of an *in vitro* model to quantify this cell death in mouse hippocampal slices subjected to oxygen-glucose deprivation (OGD), a condition mimicking cerebral ischemia in the acute brain slice. In addition to addressing the development and optimization of this model, my thesis examines its utilization to study neuroprotective strategies targeting ASIC-mediated cell death inhibition as well as the mechanism involved.

Acid-sensing ion channels were first discovered by Krishtal and Pidoplichko in 1980 when they found that neurons in the rat trigeminal and spinal ganglia displayed an inward cation current in response to a decrease in pH (Krishtal and Pidoplichko, 1980). In 1997, ASICs were cloned for the first time by Waldmann et al. and characterized as a member of the epithelial sodium channel/degenerin family of ion channels (Waldmann et al., 1997). Although first isolated in specific ganglia, it is now known that ASICs are expressed ubiquitously throughout the central and peripheral nervous systems with high levels of expression in the dorsal root ganglia, olfactory bulbs, hippocampus, amygdala, cerebellum, and cerebral cortex (Waldmann and Lazdunski, 1998). Four genes encode seven known ASIC subunits, including ASIC 1a, 1b1, 1b2, 2a, 2b, 3 and 4 (Chu and Xiong, 2012), which can form heteromeric or homomeric trimers with varying kinetics, pH sensitivities, locations, and functions (Escoubas et al., 2000). For instance, ASIC1a can be found in the brain and sensory neurons, ASIC2a is found mostly in the brain, and ASIC3 is found only in nociceptors (Escoubas et al., 2000), whereas ASIC4 is found in heterotrimers with other ASIC subunits in certain brain regions as well as in the pituitary gland (Kellenberger and Schild, 2002). Thus, ASICs are widespread channels highly expressed throughout the nervous system.

Structurally, all ASIC subunits are comprised of two transmembrane domains, a long extracellular loop containing several conserved cysteines, and shorter N- and C- termini on the intracellular surface (Chu and Xiong, 2012). ASICs are gated by protons and activated by low pH. Upon activation, the channels open to allow cation influx and are most permeable to Na^+, although they also display some permeability to Li^+, K^+, Ca^{2+}, and H^+ (Waldmann et al., 1997). Under normal circumstances, it is thought that ASICs are activated during synaptic transmission when synaptic vesicles release protons and lower the pH in the synapse (Du et al., 2014; Kreple

et al., 2014). In the presence of protons, or acidic pH, ASICs are activated and produce transient current (Vick and Askwith, 2015). The pH level needed to activate ASICs varies depending on the ASIC subunits making up the channel (Vick and Askwith, 2015), however ASICs are known to be capable of activation at pH 6.7 and below. In conditions when the pH is lower than physiological pH but not low enough to activate ASICs, the channel enters a state called steady state desensitization (Escoubas et al., 2000) and cannot open even if the pH continues to drop, which is important to the channel's normal function and ability to respond to rapid pH changes at the synapse (Vick and Askwith, 2015). Additionally, although ASICs are gated by protons, they can be affected by toxins (Vick and Askwith, 2015), and they are also normally modulated by neuropeptides, found after neurotransmitter release in the synaptic cleft (Vick and Askwith, 2015). Some of these neuropeptides slow inactivation and produce a sustained current or decrease the proton sensitivity of steady state desensitization (Askwith et al., 2000). Thus, the structure and normal molecular activity of ASICs are characterized and can be manipulated pharmacologically.

Interestingly, much of this physiological activity as well as behavior are altered by knocking out ASIC genes in mice. ASIC knockout mice were produced for the study of ASIC activity using a technique which takes advantage of homologous recombination in embryonic stem cells. In this process, a replacement vector was developed containing a gene yielding neomycin resistance flanked by regions of homologous DNA that normally flank the ASIC1 gene (Hall et al., 2009). The vector was transfected into embryonic stem cells from a mouse blastocyst *in vitro*, and homologous recombination was allowed to occur in order to replace the ASIC1 gene in the chromosomes of the stem cells with the neomycin resistance gene (Hall et al., 2009). These stem cells were injected into a mouse blastocyst and implanted into the uterus of a

mouse to obtain chimeric mice made up of some cells derived from the host blastocyst and some from the stem cells (Hall et al., 2009). Chimeric mice possessing reproductive cells with the ASIC1 gene knocked out were bred with wildtype mice to obtain mice heterozygous for the ASIC1 gene, which were bred to obtain homozygous knockout mice no longer possessing the ASIC1 gene in any of their cells (Hall et al., 2009). These mice have been used for the vast majority of studies assessing the many roles of ASICs in the nervous system.

The creation of mice genetically lacking ASIC genes has been invaluable for determining what ASICs do physiologically and how they contribute to pathological situations. For instance, it was discovered that knocking out the ASIC1 gene yields a loss of current in central neurons above pH 5 as well as problems in behaviors related to anxiety, panic, depression, and fear (Vick and Askwith, 2015). Associated with fear behaviors, learning, memory, and synaptic plasticity have been shown to be impaired in ASIC knockout mice as well (Wemmie et al., 2002). A study using high frequency stimulation found impaired long term potentiation in ASIC knockout mice (Wemmie et al., 2002). Deficits in ASIC knockout mice were also discovered with spatial learning and memory as well as with associative learning, using the hidden platform version of the Morris water maze and classical eyeblink conditioning, respectively (Wemmie et al., 2002). In contrast, studies examining cocaine-conditioned place preference in ASIC1a knockout mice and cocaine self-administration suggest that ASIC1a in the nucleus accumbens negatively regulates learning and memory associated with drug use as well as plasticity associated with addiction (Kreple et al., 2014). Such studies have indicated important physiological roles for ASICs in the nervous system and characterized the effects of removing them.

Additionally, ASIC1a has been implicated in modulation of innate fear processes (Coryell et al., 2007). A series of studies with mice lacking the ASIC1a gene showed reduced

fear behavior relative to ASIC wildtype mice in the open field test as well as reduced unconditioned acoustic startle response and reduced freezing in response to predator odor TMT (Coryell et al., 2007). Further, reduced current in amygdala neurons from ASIC1 knockout mice following acidosis implicates ASIC1 in learned fear (Wemmie et al., 2003). Investigating ASIC involvement in learned fear behaviors associated with the amygdala using an auditory fear conditioning test showed that ASIC1 knockout mice display decreased fear behavior related to cue and context fear conditioning (Wemmie et al., 2003). Thus, the use of ASIC knockout mice has been crucial in the discovery and characterization of the involvement of ASIC1 in fear, among other important normal brain functions.

Critically, in addition to carrying out key physiological functions, ASIC activity has been shown to play a role in degeneration and cell death following cerebral acidosis. Throughout the nervous system, ASICs are activated by small pH changes that occur with neurotransmitter release to perform their physiological functions (Du et al., 2014; Kreple et al., 2014). However, ASIC1a-induced neurological injury results when pH is reduced in the brain over a long period of time, which occurs in many neurological disorders and neurodegenerative diseases. In ischemic stroke, oxygen supply is cut off from the brain tissue, lowering glucose levels as well as the pH to as low as pH 6.0 (Kagansky et al., 2001). This pH reduction is largely a result of increased anaerobic respiration leading to lactic acid buildup as well as proton buildup from ATP hydrolysis (Xiong et al., 2008) and can remain for hours even after the blood supply is restored (Pignataro et al., 2007). This leads to neuronal death and brain injury through activation of ASIC1a. In a rat model of ischemia induced by middle cerebral artery occlusion (MCAO), inhibiting ASIC1a via intracerebroventricular injection of psalmatoxin-1 (PcTX), a specific ASIC1a inhibitor, reduced infarct volume by approximately 60% (Xiong et al., 2004). ASIC1

gene knockout has also been shown to provide neuroprotection following MCAO in rats (Xiong et al., 2004). Additionally, intracerebroventricular injection of sodium bicarbonate has also yielded neuroprotection, confirming that acidosis is triggering the cell death (Xiong et al., 2008). ASIC1a is an especially attractive target for preventing brain injury following ischemia because pathological acidosis is long lasting (Pignataro et al., 2007), and inhibiting ASIC1a even hours after the blood flow has been restored has been shown to prevent further injury (Pignataro et al., 2007; Vergo et al., 2011). Thus, ASICs represent a potential therapeutic target within a clinically relevant time window.

In addition to ischemic stroke, acidosis and ASICs play a role in a number of other conditions which foster brain damage. These include multiple sclerosis (Vergo et al., 2011), traumatic brain injury (Yin et al., 2013), retinal degeneration (Ettaiche et al., 2004), Parkinson's disease (Ortega-Ramírez et al., 2017), and neurodegeneration in spinocerebellar ataxia (Vig et al., 2014). In a mouse model of multiple sclerosis, ASIC1 was shown to be upregulated in axons and oligodendrocytes, and axonal damage and demyelination were decreased by exposure to amiloride, an unspecific inhibitor of ASICs (Ortega-Ramírez et al., 2017). Additionally, in a mouse model of traumatic brain injury, neurodegeneration was lessened with bicarbonate administration as well as with knockout of the ASIC1a gene (Yin et al., 2013). Similarly, mice with ataxin-1 mutations associated with spinocerebellar ataxias have shown reduced disease phenotype with knockout of the ASIC1a gene (Ortega-Ramírez et al., 2017). Thus, ASIC1a is implicated widely in neurodegenerative conditions, and therapies targeting inhibition of ASIC-mediated cell death in ischemic stroke may be useful tools capable of preventing injury in multiple other pathological conditions as well.

ASICs represent a novel potential therapeutic target for neurodegeneration as well as brain damage following ischemic stroke. This is important as there are few effective therapeutics to limit brain damage following ischemic stroke. One strategy is the use of the recombinant tissue plasminogen activator (TPA), which works by breaking up arterial clots to restore blood flow to the brain. This strategy has yielded improved neurological outcomes in a large clinical trial (Schehr, 1996) and is used today. Unfortunately this approach ultimately has limited effectiveness in preventing permanent brain damage as it can be dangerous and highly increases patients' risk of cerebral hemorrhage (Schehr, 1996). Thus, there are multiple exclusions for TPA intervention based on other morbidity factors, such as secured aneurysm or recent major surgery, as well as timing of arrival at emergency departments after stroke onset (Demaerschalk et al., 2016). Only an estimated 6-8% of stroke patients are even eligible to receive TPA therapy (Demaerschalk et al., 2016). Other neuroprotective strategies targeting distinct cell death pathways have been tried and failed to safely prevent permanent brain damage following an ischemic event. One strategy targeted voltage-gated calcium channels, but this too was ineffective in addition to causing negative cardiovascular side effects (Schehr, 1996). Similarly, the use of NMDA and AMPA antagonists have been tested in clinical trials but have also failed to provide effective neuroprotection and induced intolerable side effects such as hallucinations and cardiovascular effects (Schehr, 1996). However, inhibiting ASIC activity could reduce excitatory transmission without the side effects of NMDA antagonism, which would be consistent with the viability and fairly normal baseline synaptic transmission measured in ASIC knockout animals (Wemmie et al., 2002). ASICs represent an attractive target for neuroprotection following cerebral acidosis, however more work is needed to understand the mechanisms underlying ASIC-mediated cell death and ways ASICs can be regulated to alter

pathological activity. Further, a better understanding of interventions that might inhibit ASICs for therapeutic gain is needed before strategies targeting ASICs can be fully developed.

Several ASIC inhibitors exist and are used to study ASIC activity, regulation, and neuroprotection. Amiloride acts as a diuretic which blocks sodium ion exchangers and the epithelial sodium channel (ENaC) as well as ASICs (Xiong et al., 2008). Amiloride is thought to inhibit ASIC activity by blocking the channel pore and was shown to reduce ASIC-mediated pain and ischemic neuronal death (Xiong et al., 2008). However, amiloride is not viable as an inhibitor of ASIC-mediated cell death in humans as its non-specificity leads to inhibition of ion exchange systems that are critical for survival (Xiong et al., 2008). Similarly, A-317567 is a non-selective blocker of ASIC current at low doses that shows promise in alleviating ASIC-mediated chronic pain, although it minimally crosses the blood brain barrier and may affect the activity of vital ion exchange systems (Xiong et al., 2008). In addition to preventing prostaglandin production, non-steroid anti-inflammatory drugs (NSAIDS) have also been shown to inhibit ASIC activity and sensory neuron ASIC expression in inflammation, suggesting effectiveness of NSAIDS in the inhibition of painful conditions involving acidosis in the periphery, such as inflammation, infection, and blisters (Xiong et al., 2008). However, the concentration of NSAIDS required to inhibit ASIC activity is terrifically high and not viable to therapeutic intervention. Thus, there are no small molecule inhibitors of ASICs that can be used to specifically target ASIC activity in humans.

One other strategy for ASIC inhibition relies on the use of venom peptides. Critically, certain peptides from venoms have shown to be useful tools in isolating ASIC activity and studying neuroprotection in ischemia. The peptide MitTx from the venom of the Texas coral snake acts as an ASIC agonist to induce pain (Bohlen et al., 2011), whereas mambalgins from

the venom of the black mamba snake (Diochot et al., 2012) and the peptide toxin APETx2 from the sea anemone (Xiong et al., 2008) block pain through ASIC inhibition. Psalmotoxin-1 (PcTX), a peptide toxin from the venom of a South American tarantula, inhibits homomeric ASIC1a channels as a gating modifier with high specificity at nanomolar concentrations (Xiong et al., 2008). Intracerebroventricular injection of PcTX in animal models of ischemia has shown to be neuroprotective within a 5 hour time window (Xiong et al., 2008). However, PcTX may present problems in translation to human usage as its structure as a large peptide with three disulfide bonds makes it both difficult to synthesize and to cross the blood brain barrier (Xiong et al., 2008). Also the actions of PcTX with long term exposure are unclear. Its disulfide bonds may decrease stability due to sensitivity to oxidation and reduction (Xiong et al., 2008). There is also evidence from preliminary electrophysiology studies from the Askwith lab that long term exposure to PcTX enhances glutamatergic synaptic transmission and excitotoxicity. Additionally, the involvement of ASIC1a in important normal brain functions may compromise the advantages of this approach of complete ASIC inhibition to regulate ASIC-mediated neuronal death (Xiong et al., 2008). Overall, current ASIC inhibitors present problems and challenges in translation to human use in neuroprotection, and no strategies or treatments exist to specifically inhibit ASIC1a in patients. Identification of additional compounds and mechanisms targeting ASIC-mediated cell death inhibition are needed in order to develop an effective treatment to prevent debilitating brain injury following cerebral acidosis, potentially using existing therapeutics which may regulate ASIC activity.

Traditionally, the Askwith lab employs mechanisms to study ASIC involvement in cell death in the hopes that new strategies to target ASICs can be developed. The Askwith lab has mainly worked in tissue culture and whole animal studies. In cell culture, ASIC-mediated cell

death can be studied using assays exposing cells to a low pH solution (e.g. pH 6.0) versus a solution at physiological pH (pH 7.4). This approach has several advantages for conducting studies of the fundamentals of ASIC activity and cell death. For one, cells are all from one type of tissue or cell line (e.g. CHO cells or hippocampal neurons), so experiments are controlled to study the response of cells of interest. Additionally, cell growth and extracellular conditions are closely controlled, and ASIC activity can be easily isolated in a cell line lacking ASICs through ASIC transfection. However, drawbacks of this approach are that cells are removed from their native tissues so they lack natural cellular connections and have altered microenvironments when they are grown homogenously on a dish in cell culture media. Neurons cultured on a dish, for instance, lack interactions with other glial cells and neuron types that normally could be affecting their activity in the native brain environment. In addition, these tissues must come from neonatal animals. Further, acidosis solutions miss aspects of the conditions surrounding cells during cerebral deprivation of oxygen and glucose, and cells and slice cultures often must incubate for prolonged time periods before cell death can be measured. In contrast, whole animal studies leave native brain conditions and cellular connections for the most part intact. As a result, animal models of focal ischemia, such as middle cerebral artery occlusion, are useful to study actual effects in the mouse brain of ASIC inhibition. However, these models present challenges as individual factors, mechanisms, timing, and responses are more difficult to control and measure. Moreover, procedures are often invasive and technically highly challenging. Overall, a better system to study ASIC involvement in cell death eliminating some of these major challenges and bridging tissue culture and whole animal studies is needed.

One such system is the *in vitro* slice. The utilization of brain slices to study ASIC involvement in cell death has the advantages of maintaining many cellular connections and

conditions present in the native environment of the neurons under study in a controlled model that can be visualized. Although brain slices may exhibit increased sensitivity to ischemia and transcription of stress-related mRNA following the action of slicing (Lipton, 1999), slices represent valuable tools leading to *in vivo* studies as they maintain much of the cytoarchitecture of the brain and can be utilized without performing demanding brain surgery on live animals or introducing blood brain barrier and whole animal factors. Slice cultures are valuable tools normally used to study processes that occur over long periods of time as they can be maintained for months, whereas acute slices are widely used for short-term same-day electrophysiological studies (Lein et al., 2011). Specifically hippocampal slices have been widely used in electrophysiological studies following ischemia in oxygen-glucose deprivation (Lipton, 1999). Notably, slice cultures from rat brains were first used to quantify and study OGD induced cell death itself in 1995 by Strasser and Fischer (Strasser and Fischer, 1995). More recently, acute slices were shown to be useful tools in measuring ASIC-mediated cell death in oxygen-glucose deprivation (Bhowmick et al., 2017). Importantly, with the use of acute slices, cell death can be measured relatively immediately following the cell death event, and this cell death can be readily visualized.

The acute brain slice subjected to oxygen-glucose deprivation represents a vital *in vitro* model to quantify ASIC-mediated cell death and explore neuroprotective interventions bridging cell culture and whole animal studies. In this thesis, I attempt to implement this in the Askwith lab through the development and optimization of an acute mouse brain slice model to quantify OGD-induced cell death mediated by acid-sensing ion channels. This model will provide increased replicability of the brain's natural environment during cerebral acidosis compared to our neuronal culture studies. Finally, I will utilize this model to test novel therapeutics that

potentially inhibit ASIC activity and examine a molecular hypothesis on the mechanism of ASIC-mediated cell death.

Chapter 2: Method Development

Introduction

The problem with targeting ASICs directly is that there are no existing therapeutics that function by directly inhibiting ASIC1a. Thus, testing the therapeutic potential of ASICs in humans has not yet been accomplished. The Askwith lab is focused on understanding how ASIC1a and acidotoxicity are regulated in order to determine whether strategies limiting ASIC-induced toxicity could be developed from existing therapeutics. This regulation is studied in the lab using mainly neuronal cultures and electrophysiology. However the goal of the work covered in this chapter is to develop and optimize a method to quantify ASIC-medicated cell death in hippocampal slices subjected to oxygen-glucose deprivation (OGD), mimicking the conditions in the brain associated with cerebral ischemia. This will allow us to better mimic cerebral ischemia in an *in vitro* situation and provide a bridge between using neuronal culture and whole animal studies.

Using this model, we can also add interventions of compounds of interest (Chapters 4 and 5) and measure cell death to determine if a compound interacts with the ASIC-mediated cell death pathway to yield neuroprotection and to elucidate the mechanism of ASIC mediated cell death. The protocol development and optimization for the methods used to induce and quantify ASIC-mediated cell death in mouse hippocampal slices subjected to oxygen-glucose deprivation are outlined in this chapter.

Methods

Animals

All experiments with vertebrate animals and animal protocols have been approved by the Institutional Animal Care and Use Committee at The Ohio State University. C57 black 6 male mice between the ages of 21-32 days from our transgenic mouse colony were sacrificed for use in this model.

Solutions

Cutting Solution: NaCl (125 mM), $NaHCO_3$ (25 mM), NaH_2PO_4 (1.25 mM), KCl (3.5 mM), $CaCl_2$ (0.1 mM), $MgCl_2$ (3 mM), and Glucose (10 mM). Chilled on ice and bubbled with 95% O_2 5% CO_2 for 45 minutes prior to use.

Artificial Cerebrospinal Fluid (aCSF): NaCl (125 mM), $NaHCO_3$ (25 mM), NaH_2PO_4 (1.25 mM), KCl (3.5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), and Glucose (10 mM). Kept at room temperature and bubbled with 95% O_2 5% CO_2 for 45 minutes prior to use.

Oxygen-Glucose Deprivation (OGD) Solution: NaCl (125 mM), $NaHCO_3$ (25 mM), NaH_2PO_4 (1.25 mM), KCl (3.5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), and Sucrose (10 mM). Kept at room temperature and bubbled with 95% N_2 5% CO_2 for 45 minutes prior to use.

Slicing

Mice were anesthetized with isoflurane and immediately sacrificed by decapitation to obtain the fresh brain tissue. The brain was carefully dissected out, cut to separate the hemispheres, and positioned on a vibratome bathed in ice cold oxygenated cutting solution (**Fig. 1**). The vibratome blade was set to move at 0.14 mm/s and to cut the brain into 250 µm thick slices containing the hippocampus (n=6-8 slices from one animal). Following slicing, slices were transferred to a recovery solution to equilibrate for 60 minutes in room temperature artificial cerebrospinal fluid rich in glucose and bubbled with 95% O_2 5% CO_2. Slices were held in uniquely designed holding chambers, crafted in 100-mL beakers with mesh near the bottom to gently hold slices and tubing to insert the oxygenation tube and create oxygen flow circulating through the solution. Each holding chamber contained 80 mL solution and up to four slices. It was experimentally determined that the optimal time from decapitation to the beginning of recovery was under 12 minutes in order to avoid excess cell death.

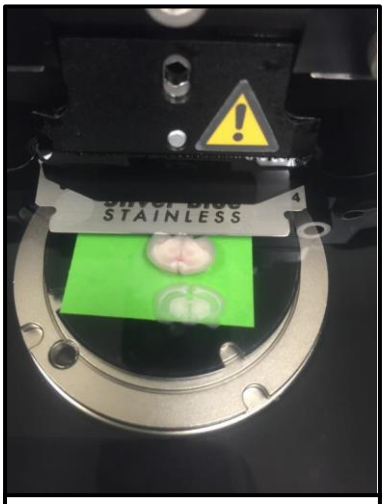


Fig. 1. Mouse Brain on Vibratome.

Treatment

Following recovery, slices were transferred to either a holding chamber containing glucose rich, oxygenated aCSF (control) or one containing solution mimicking cerebral oxygen-glucose deprivation with glucose replaced by sucrose and oxygen gas replaced by nitrogen gas. The slices were subjected to treatment/control for precisely 20 minutes.

Reperfusion Mimic

Following the period of oxygen-glucose deprivation or control, the slices were allowed to recover in aCSF once again containing glucose and oxygen for 60 minutes (**Fig. 2**). This reperfusion mimic was experimentally determined to aid in discrimination between the OGD-treated slices and the control slices in this model of ischemia as it allowed the OGD-exposed slices necessary time to carry out the cell death induced by the 20 minute OGD treatment.

Fig. 2. Holding chamber containing slices bathed in artificial cerebrospinal fluid and bubbling with 95% O_2 5% CO_2 gas.

Visualizing Cell Death

Following the reperfusion mimic, the slices were exposed to TO-PRO®-3Iodide stain for 20 minutes. We chose this far red stain as it labels dead cells by entering the dead cells through their compromised cell membranes and intercalating with their DNA (**Fig. 3**), causing dead cells to fluoresce under a confocal microscope. Following staining, slices were briefly rinsed in aCSF to wash off excess stain and then fixed in 4% paraformaldehyde shaking on a twelve well plate for

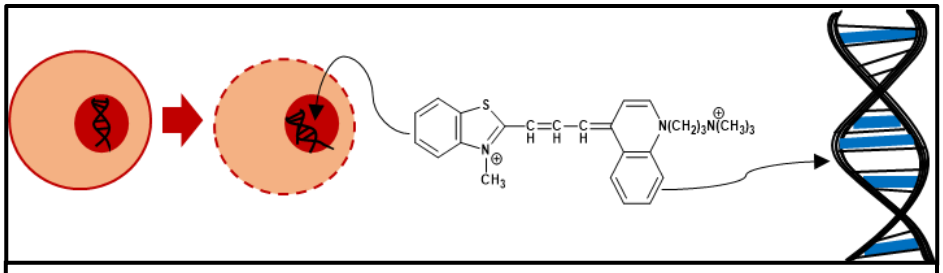


Fig. 3. TO-PRO®-3Iodide Mechanism. Stain enters dead cells through the compromised cell membrane and intercalates with DNA, allowing dead cells to be visualized by fluorescence in the far red spectrum.

60 minutes. Fixed slices were then mounted on microscope slides coated in Immuno Mount and allowed to cure for 45 minutes before imaging.

A confocal microscope within the Department of Neuroscience was used to image the slices at the CA1 pyramidal cell layer of the hippocampus. It was experimentally determined that imaging the slices at a depth of 25 µm within the tissue was optimal for visualization of a stained, in-tact pyramidal cell layer and to discriminate between OGD-induced cell death and baseline cell death in the aCSF control (**Fig 4**).

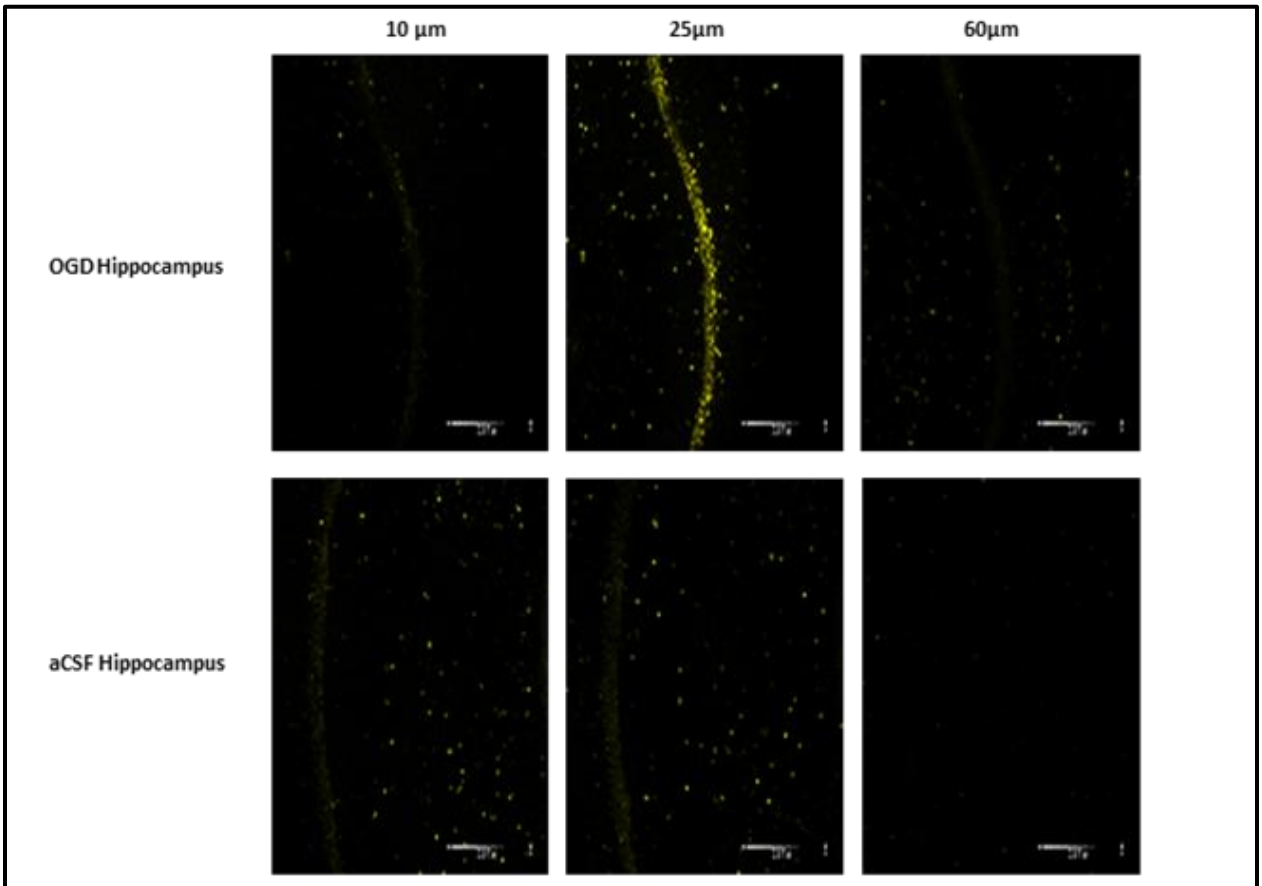


Fig. 4. Depth and Cell Staining. Images were taken at different depths within the brain slice to determine the optimal depth of visualization. Dead cells are represented by yellow fluorescence in the images. As shown by comparing the middle column of images at 25 µm within the slice to the right and left columns, optimal discrimination between the treatment groups occurs at 25 µm deep.

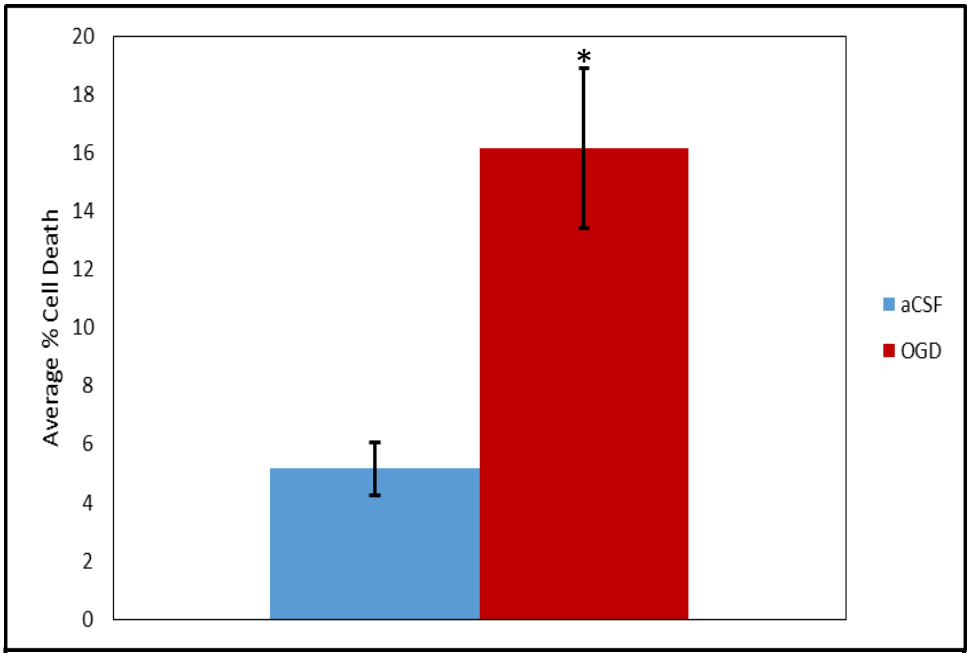
Quantification/Statistics

Cell death, represented by fluorescence in images taken using the confocal microscope, was quantified for OGD-exposed slices and control slices using histogram analysis through a program called FIJI. Thresholds were set for the intensity of fluorescence of pixels representing living and dead cells. These thresholds could be used to calculate the percentage of cell death within a specific area size of the pyramidal cell layer of the hippocampus in each brain slice. The average percent cell death for slices in the aCSF control group and slices in the OGD group were calculated, with n=1 for slices from one animal.

Statistical significance was determined using standard methods in the field (shown in the graph descriptions), with a p value less than 0.05 indicating statistical significance. To compare two groups, we used an unpaired t-test when appropriate. When more than two groups were compared, a one-way ANOVA with Bonferroni Post Hoc analysis were used to determine statistical significance between control and intervention.

Results

With the procedure outlined above optimized for all conditions and variables, cell death data was collected using seven ASIC wildtype animals. Data from OGD-exposed slices from each animal were averaged, and data from control slices from each animal were averaged. Average percent cell death in slices exposed to oxygen-glucose deprivation and control slices was compared



Graph 1. ASIC WT, OGD vs. Control. Wild type mouse hippocampal brain slices were exposed to oxygen-glucose deprivation (OGD) or control (aCSF). Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. One mouse counts as a sample, with n=7. Bars represent hippocampal cell death averaged across the samples (unpaired t-test p value= 0.0026).

(**Graph 1**). Cell death was significantly lower in the aCSF control than in the OGD-exposed slices (p value=0.0026). These results indicate that this model can be used to both induce cell death by oxygen-glucose deprivation in mouse hippocampal slices and to quantify this cell death.

Conclusion

Data collected from the development and optimization of this model shows its usefulness as an *in vitro* model to study OGD-induced cell death and how it can be regulated. This model is a crucial bridge between studies in neuronal cultures and whole animal studies as it provides a way

to study cerebral acidosis in an *in vitro* situation which better mimics the native conditions within the brain during ischemia. Moving forward in this thesis, this model will be used with interventions of various compounds of interest from previous studies in the lab and from the literature to determine how the cell death measured using this model can be changed and regulated and to further elucidate a possible mechanism of ASIC-mediated death in cerebral ischemia. Importantly, in the next chapter, I will first discuss the ASIC1 specificity of this model determined by running trials with specific pharmacological ASIC1 inhibition as well as genetic knockout of the gene encoding the ASIC1 subunit.

Chapter 3: Pharmacological Inhibition and ASIC1 KO

Introduction

Mice genetically lacking the ASIC1a subunit have shown deficits in pain, fear behaviors, and learning (Wemmie et al., 2002). Importantly, ASIC1a is the major ASIC subunit involved in ischemic neuronal death. Therefore, the OGD-induced cell death in our brain slice model of ischemic stroke can be tested for ASIC1 specificity by running trials of the experiment using ASIC1 KO mice and measuring cell death. The rationale for these studies is that, if cell death is equal in OGD-exposed slices and control slices in brain slices lacking the ASIC1 subunit, then there would be evidence that the cell death observed in this model is mediated by ASIC1.

Similarly, measurements of cell death in brain slices exposed to oxygen-glucose deprivation in the presence of a pharmacological ASIC1 inhibitor are also useful to determine the specificity of this *in vitro* model to ASIC-mediated cell death. Specifically, we used a toxin, called Psalmotoxin-1 (PcTX), purified from the venom of a South American tarantula,

Psalmopoeus cambridgei (Escoubas et al., 2000). This toxin has been shown to inhibit homomeric ASIC1a channels with high specificity and affinity (Escoubas et al., 2000), making it a useful compound to help characterize the cell death pathways occurring in the OGD-induced cell death of our model. If cell death is reduced to basal levels in wildtype mouse brain slices exposed to oxygen-glucose deprivation with the ASIC1 inhibitor, PcTX, the observed cell death can be understood to be ASIC-mediated.

Methods

Pharmacological Inhibition

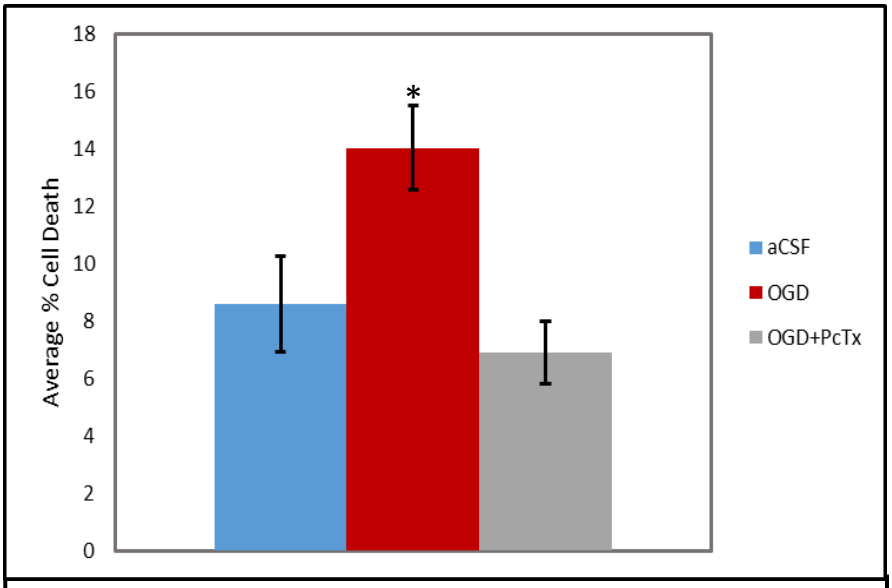
The *in vitro* hippocampal slice model developed and described through Chapter 2 above was carried out following the same protocols for animals, solutions, slicing, recovery, treatments, reperfusion, staining, fixing, imaging, and quantification. The difference in these trials was that an intervention of PcTX was added acutely during the 20-minute OGD period. Because of this new treatment group, there were three groups of slice treatments: OGD, OGD plus PcTX (100 nM), and an aCSF control. Slices were treated the same as in the model described in Chapter 2, and cell death was compared between each of the three treatment groups

ASIC1 Knockout

The model described in Chapter 2 was used in the same manner, except slices were obtained from ASIC1 knockout mice instead of ASIC wildtype mice.

Results

Decreased cell death was observed in OGD-exposed slices with acute administration of Psalmotoxin-1 (**Graph 2**). The average level of cell death in the OGD+PcTX group in the pyramidal cell layer of the hippocampus was reduced completely to the baseline level of cell death observed in the control group, while cell death in the OGD group was approximately twice as high (**Fig 5**).



Graph 2. ASIC WT, OGD vs. OGD+PcTX vs. Control. Wild type mouse hippocampal brain slices were exposed to oxygen-glucose deprivation (OGD), control (aCSF), or OGD+PcTX. Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. One mouse counts as a sample, with n=6. Bars represent hippocampal cell death averaged across the samples (one-way ANOVA p value=0.0076). Bonferroni's Multiple Comparison test showed aCSF vs. OGD P <0.05, aCSF vs.OGD+PcTx P >0.05, OGD vs. OGD+PcTx P <0.01.

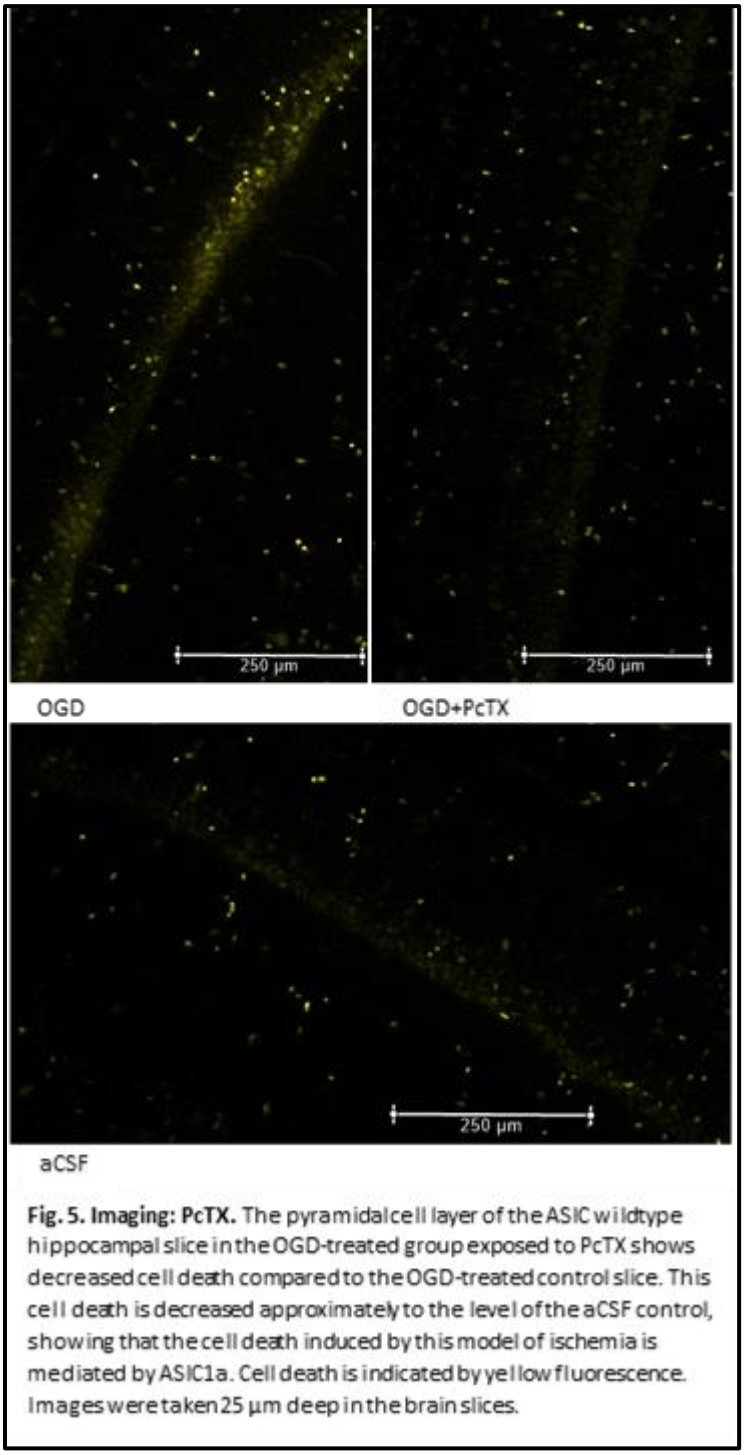
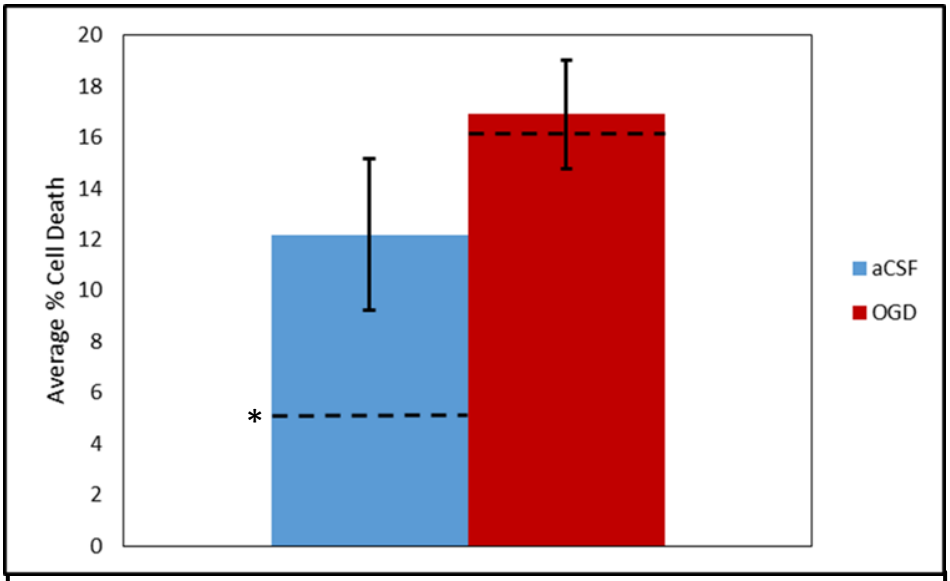


Fig. 5. Imaging: PcTX. The pyramidal cell layer of the ASIC wildtype hippocampal slice in the OGD-treated group exposed to PcTX shows decreased cell death compared to the OGD-treated control slice. This cell death is decreased approximately to the level of the aCSF control, showing that the cell death induced by this model of ischemia is mediated by ASIC1a. Cell death is indicated by yellow fluorescence. Images were taken 25 µm deep in the brain slices.

This was observed with slices from six animals (n=6, p value=0.0076) and 6-8 slices per animal. This suggests that the cell death being induced and quantified using this model is mediated by ASIC1.

As expected, initial trials run with three ASIC1 KO mice showed no significant difference in levels of cell death between the OGD-exposed slices and control slices (p value=0.26, **Graph 3**). However, unexpectedly, basal cell death was increased in the control slices from the ASIC1 KO brain compared to basal cell death in the ASIC wildtype (p value=0.015, **Graph 3**). This increased level of cell death in the knockout mouse brain slice can be observed in the hippocampus of ASIC1 knockout mouse brain slices exposed to OGD or an aCSF control (**Fig. 6**). Basal cell death was significantly higher in the ASIC1 KO control than in the wildtype control, indicating a possible compensatory mechanism occurring in the ASIC1 knockout brain yielding excessive



Graph 3. ASIC1 KO, OGD vs. Control. Knockout mouse hippocampal brain slices were exposed to oxygen-glucose deprivation (OGD) or control (aCSF). Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. One mouse counts as a sample, with n=3. Bars represent hippocampal cell death averaged across the samples (p value= 0.26). Wild type data is represented by dashed lines, showing an increase in basal cell death in the ASIC1 knockout (unpaired t-test p value= 0.015).

cell death in the hippocampus following slicing compensating for chronic absence of the ASIC1 subunit. This compensation could be mediated by excess glutamate and excitoxicity in the absence of ASIC1.

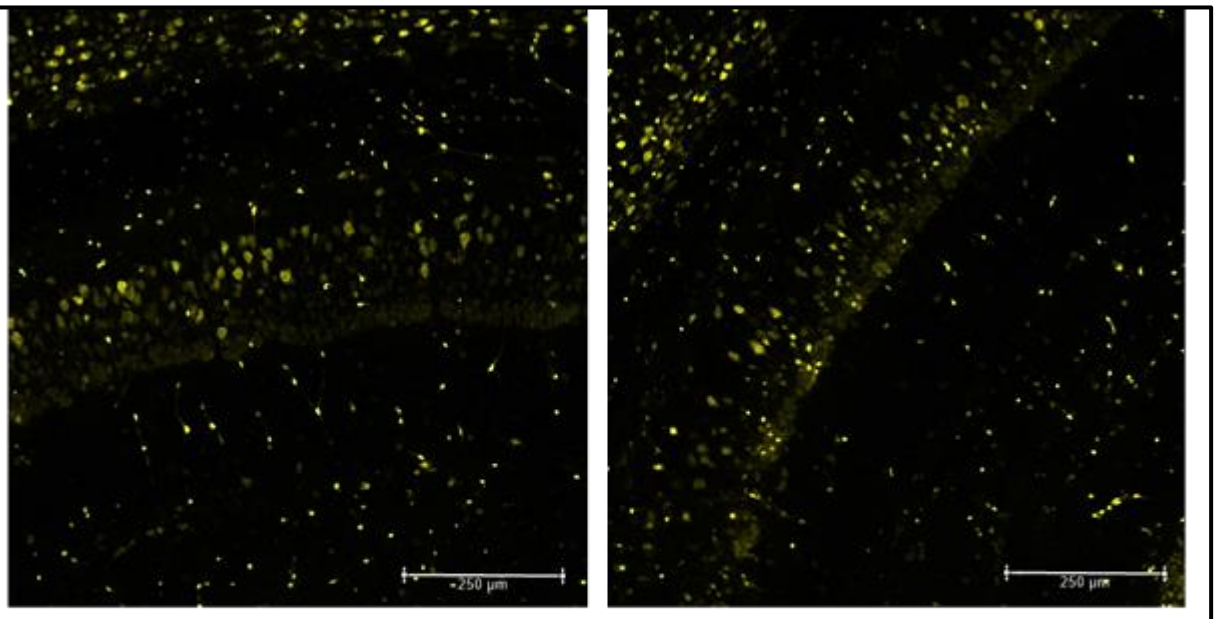
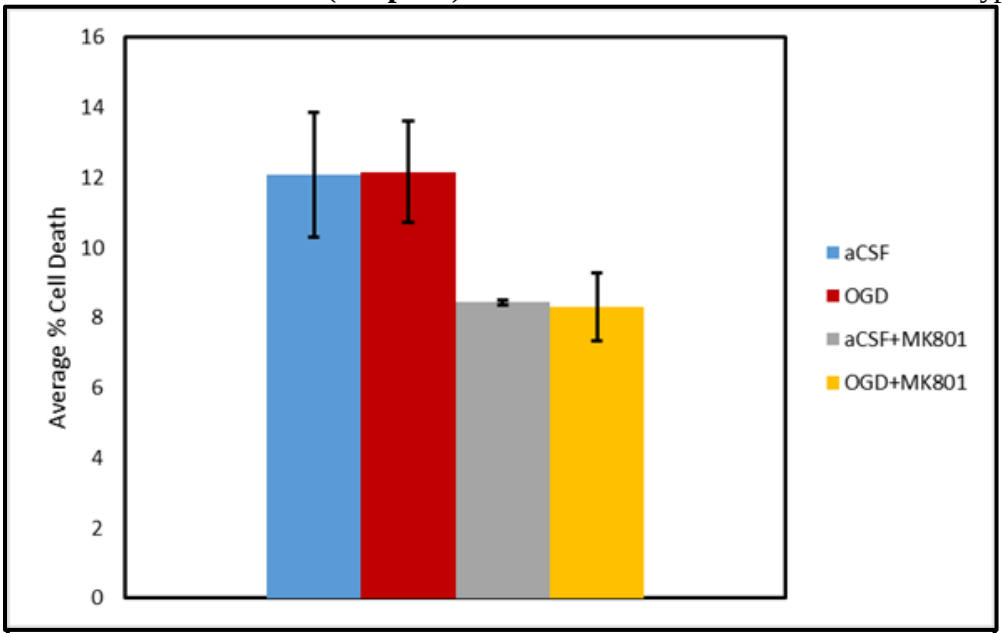


Fig. 6. Imaging: ASIC1 KO. Observing the pyramidal cell layer in ASIC1 knockout hippocampal slices reveals increased cell death compared to the wildtype, indicated by increased yellow fluorescence of cells. Images were taken 25 µm deep in the brain slices.

This hypothesis was tested by adding an intervention of the N-Methyl-D-aspartate (NMDA) receptor antagonist, MK801, to the recovery, treatment, and reperfusion mimic stages of both the OGD and control groups in our *in vitro* slice model with ASIC1 knockout mice. Additional cell death data was collected from ASIC1 knockout mouse hippocampal slices exposed to OGD, aCSF, OGD+MK801, or aCSF+MK801 (1 μM).

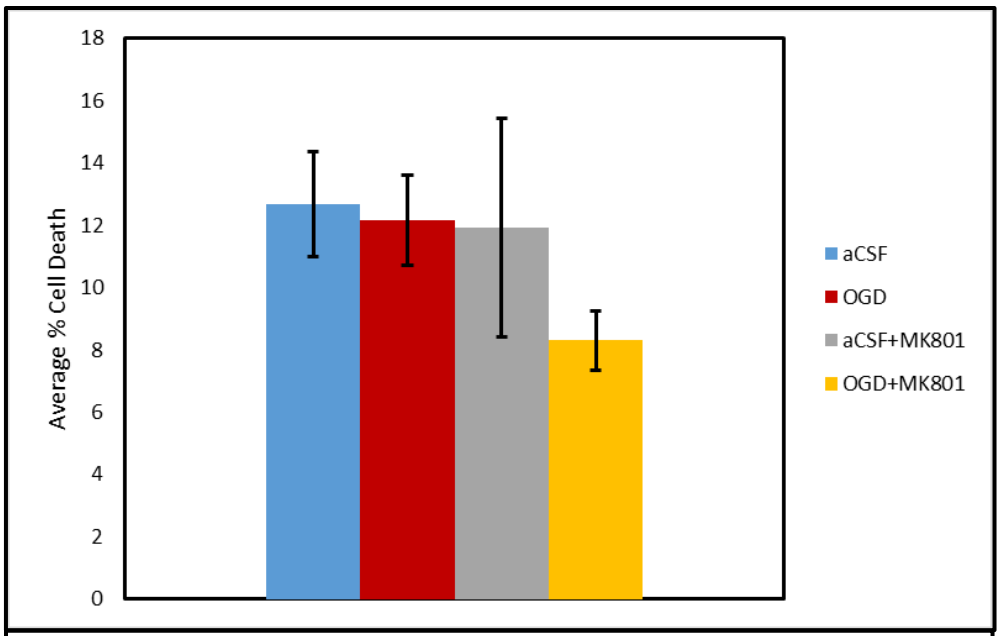
With an increased sample size, cell death levels in ASIC1 KO slices were measured to be nearly identical following either OGD exposure or control (**Graph 4**), while still elevated above cell death levels seen in ASIC wildtype mice. With the addition of MK801, the NMDA antagonist, to the ASIC1 KO slices, we saw a trend of decreased cell death levels in the OGD-exposed slices as well as the control slices (**Graph 4**) to near the levels seen in the ASIC wildtype aCSF control.



Graph 4. ASIC1 KO, OGD vs. aCSF vs. aCSF+MK801 vs. OGD+MK801. ASIC1 knockout mouse hippocampal brain slices (with and without exposure to the NMDA antagonist MK801) were exposed to oxygen-glucose deprivation (OGD) or control (aCSF). Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. Bars represent hippocampal cell death averaged across the samples. One ASIC1 knockout mouse counts as a sample (aCSF/OGD n=8, aCSF+MK801/OGD+MK801 n=2, OGD/OGD+MK801 n=1, aCSF/aCSF+MK801 n=1). Significance was determined using one-way ANOVA (p value=0.37) and Bonferroni's Multiple Comparison test (P >0.05 for comparisons of each group). No significant difference was found between any of the groups. Data was excluded here from one mouse brain sample exposed to aCSF/aCSF+MK801 as cell death from one slice was approximately 3 standard deviations above the mean (See data in **Graph 5**).

The result that cell death levels are approximately equal between aCSF and OGD treatment groups in either case in ASIC1 KO slices (p value >0.05) indicates that the OGD-induced cell death we are measuring with this model is ASIC1-mediated. Additionally, we see a trend of a decrease in cell death observed in the ASIC1 KO in the presence of MK801, although not statistically significant, (p value >0.05) suggesting that the enhanced basal cell death observed in the knockout hippocampus may be due, at least in part, to an NMDA-mediated increase in excitotoxicity in mice chronically lacking the ASIC1 subunit.

Additionally, cell death data from one mouse (n=1) was excluded above (**Graph 4**) as the level of cell death measured in one single slice exposed to aCSF+MK801 was over 3 standard deviations from the mean. This excluded data can be viewed together with all of the ASIC1 knockout data previously described (**Graph 5**). Besides the slice with cell death measured over 3



Graph 5. ASIC1 KO, OGD vs. aCSF vs. aCSF+MK801 vs. OGD+MK801. Data point over 3 standard deviations from the mean included. Bars represent hippocampal cell death averaged across the samples. One ASIC1 knockout mouse counts as a sample (aCSF/OGD n=8, aCSF+MK801/OGD+MK801 n=2, OGD/OGD+MK801 n=1, aCSF/aCSF+MK801 n=2). One-way ANOVA showed p value=0.63. Bonferroni's Multiple Comparison test showed P >0.05 for comparisons of each group. Percent cell death in one single slice exposed to aCSF+MK801 was measured to be 46%, over 3 standard deviations from the mean. This data sample is included here but was excluded in Graph 4 above. Exclusion of this data from Graph 4 is further described below.

standard deviations from the mean, all other hippocampal slices in the excluded mouse brain sample had cell death levels within 1 standard deviation of the mean. Therefore, it is possible that the outlier slice was damaged in the process of transfer between solutions as this data point differed so dramatically from the rest. However, slices in this specific brain sample exposed to aCSF+MK801 did show a trend of increased cell death relative to the cell death measured in the rest of the slices exposed to aCSF+MK801 (unpaired t-test p value=0.00043). Thus, error in this sample may be due to reagent degradation as this sample was from the last experiment run with the prepared MK801 solution, which is suspected to have gone through extra freeze-thaw cycles due to freezer malfunction.

Conclusion

The results of the experiments discussed in this chapter confirm that our *in vitro* model of ischemia, described in detail in Chapter 2, is inducing cell death mediated by ASIC1 with exposure to oxygen-glucose deprivation. This is significant as it shows that this model of cerebral ischemia can be used effectively as a tool to study the regulation of cell death mediated by ASIC1 and to help elucidate the mechanism of ASIC-mediated death by targeting different parts of the hypothesized pathway. Moving forward, and discussed in the next chapter, this model can be used with interventions of suspected neuroprotective compounds to determine how they impact OGD-induced cell death in an ASIC-dependent way. In addition to building on the fundamental understanding of ASIC-mediated cell death, these studies may one day lead to new treatments to prevent much of the permanent brain damage following an ischemic event in patients.

Chapter 4: Neuroprotective Intervention: DOR Activation

Introduction

The Askwith lab has discovered that activation of the G-protein coupled receptor, delta opioid receptor (DOR), attenuates ASIC1a-induced acidotoxicity in cell culture models of acid-induced neuronal death. Opioid receptors are commonly associated with the mu opioid receptor due to its well-known involvement in pain and drug addiction. However, there are two other opioid receptors, delta and kappa, whose functions are not as well defined. The delta opioid receptor (DOR) is abundantly expressed throughout the brain, and its activation has been shown to attenuate cell death in several experimental models, including ischemia (Chao et al., 2012; Charron et al., 2008; Kao et al., 2008; Oeltgen et al., 2006; Sherwood and Askwith, 2009). The Askwith lab found that DOR activation in neuronal culture acidosis assays reduced ASIC1a-induced hippocampal neuronal death by 85%, suggesting that ASIC-induced neuronal death is central to DOR's mechanism of action in ischemia.

DOR agonists could be especially useful as a potential therapeutic for ASIC1a-induced brain injury as existing DOR agonists have already undergone clinical trials for major depressive disorder and anxiety (Hudzik et al., 2011; Pradhan et al., 2011). However, no clinical studies have tested the role of DOR-selective agonists in ischemia. The observation that DOR activation prevents ASIC1a-mediated injury would provide a molecular mechanisms for DOR-induced neuroprotection and suggest that DOR agonists target a novel mechanism of neuronal death (acidotoxicity) distinct from most strategies which have failed in clinical trials. Such a result could yield novel strategies to prevent neuronal death in humans and neurological injury in a variety of pathological conditions.

Interestingly, the Askwith lab has preliminary data that the mechanism of DOR-induced neuroprotection is unconventional. Using whole cell patch clamp neuronal analysis, the Askwith lab found that the DOR agonist, SNC80, does not alter the amplitude of the ion current or calcium influx of ASIC1a despite its preventative effects on ASIC-induced cell death. This suggests that DOR activation may prevent the toxic actions of ASIC1a, while leaving the physiological function of the channel untouched. This has large implications for possible treatments for ASIC-mediated cell death which prevent brain damage but allow the patient to retain normal ASIC functions. Thus, DOR activation may represent a novel strategy to target ASIC1a-induced cell death, offering therapeutic advantages if complete inhibition of ASIC1a function proves intolerable.

Yet, DOR activation has only been shown to limit ASIC1a-induced neuronal death in culture models of acidotoxicity. The hypothesis of this chapter's study is that DOR activation prevents ASIC1a-induced neuronal death following ischemic conditions in which acidosis is allowed to develop naturally. The goal of this chapter is to determine how the DOR agonist, SNC80, impacts ASIC1a-induced cell death in conditions similar to those following ischemic stroke using our OGD mouse brain slice model. The results of this work could help support therapeutics targeting DOR activation to prevent brain injury not only following stroke, but also other disorders involving ASIC1a-induced neuronal death, including multiple sclerosis (Vergo et al., 2011), traumatic brain injury (Yin et al., 2013), spinocerebellar ataxia (Vig et al., 2014), and retinal degeneration (Ettaiche et al., 2004).

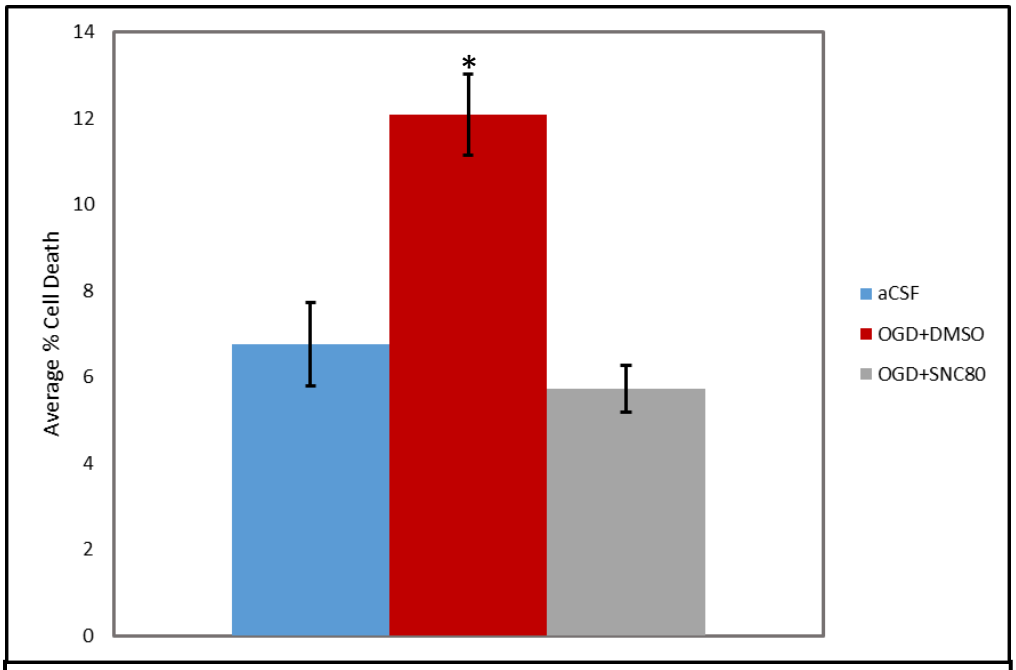
Methods

The *in vitro* hippocampal slice model of ischemia developed and described through Chapter 2 was carried out following the same protocols for animals, solutions, slicing, recovery, treatments,

reperfusion, staining, fixing, imaging, and quantification. The difference in these trials was that an intervention of SNC80 (a delta opioid receptor agonist) or DMSO was added acutely during the 20-minute OGD period. There were three groups of slice treatments: OGD plus SNC80 (1 μM), OGD plus DMSO (same volume as SNC80), and an aCSF control. Slices were treated the same as in the model described in Chapter 2, and cell death was compared between each of the three treatment groups.

Results

By comparing average percent cell death levels between the aCSF control, the OGD+DMSO control, and the OGD+SNC80 treatment (**Graph 6**), a very similar picture to the PcTX data



Graph 6. ASIC WT, OGD+DMSO vs. OGD+SNC80 vs. Control. Wild type mouse hippocampal brain slices were exposed to OGD+DMSO, OGD+SNC80, or control (aCSF). Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. One mouse counts as a sample, with n=11. Bars represent hippocampal cell death averaged across the samples (one-way ANOVA p value= 0.000015). Bonferroni's Multiple Comparison test showed aCSF vs. OGD+DMSO P <0.001, aCSF vs. OGD+SNC80 P >0.05, OGD+DMSO vs. OGD+SNC80 P <0.001.

(**Graph 2**) was observed. Basal cell death and cell death resulting from DOR activation in the OGD+SNC80 treatment group were observed to be approximately half the average percent cell

death measured in the OGD+DMSO group of slices (**Fig 7**). These data were collected with 11 ASIC wildtype mice (n=11, p value=0.000015).

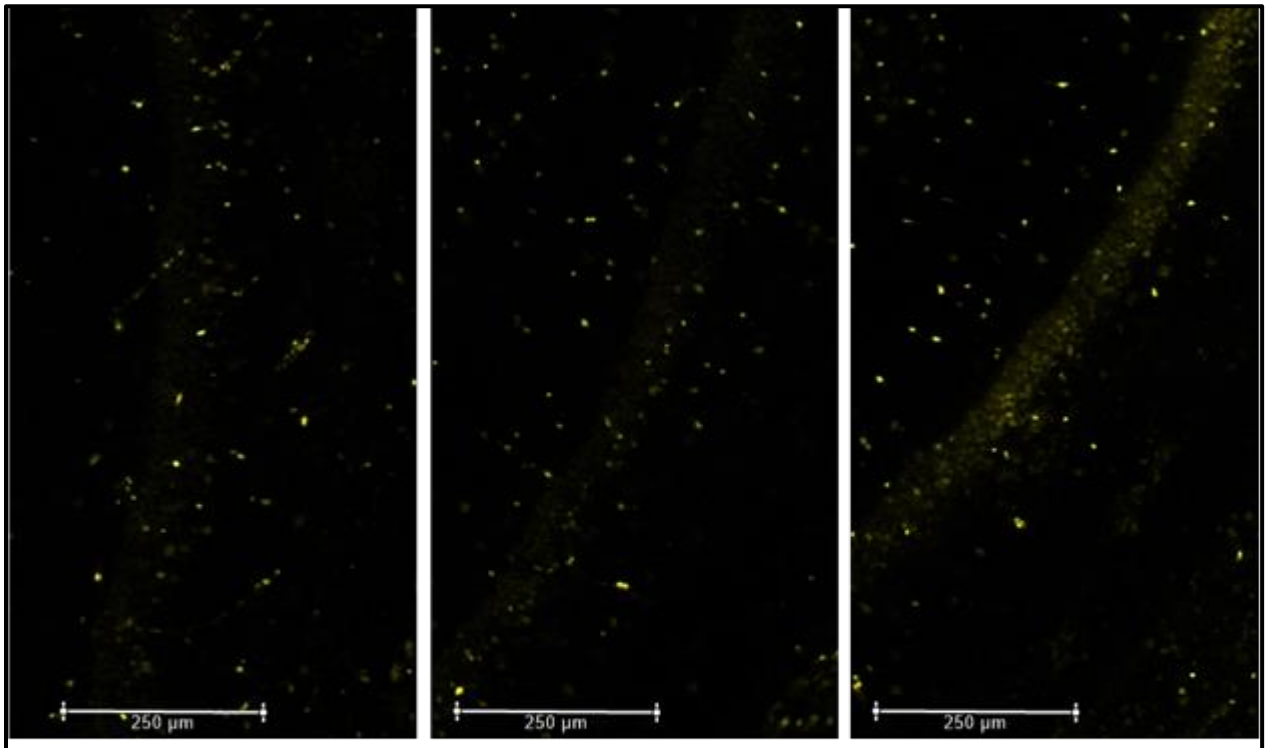


Fig. 7. Imaging: DOR Activation. The pyramidal cell layer of the ASIC wildtype hippocampal slice in the OGD-treated group exposed to SNC80 shows decreased cell death compared to the OGD-treated slice exposed to DMSO alone. This cell death is decreased approximately to the level of the aCSF control, suggesting that DOR activation by SNC80 provides complete neuroprotection to oxygen-glucose deprivation in this model. Cell death is indicated by yellow fluorescence. Images were taken 25 µm deep in the brain slices.

Conclusion

Reduction of cell death to basal levels in the mouse hippocampal slices exposed to oxygen-glucose deprivation with delta opioid receptor activation suggests that the activation of DOR may be neuroprotective in cerebral ischemia. As cell death is reduced to basal levels with DOR activation as with the addition of PcTX in OGD-exposed slices, this suggests that DOR is acting on an ASIC1-dependent pathway to induce neuroprotection. This implicates existing DOR

agonists as novel therapeutics for ischemic stroke and other neurological disorders through the inhibition of ASIC1-dependent cell death. To further elucidate the mechanism of this ASIC-mediated cell death pathway and how DOR could be exerting its neuroprotective benefits, the impact of Receptor Interacting Protein Kinase 1 (RIP1) inhibition on ASIC-mediated cell death in this model was tested. This is discussed in detail and is the main focus of the next chapter.

Chapter 5: Mechanistic Component: RIP1 Inhibition

Introduction

There is strong evidence for the involvement of the Receptor Interacting Protein Kinase 1 (RIP1) in the ASIC-mediated cell death pathway under study. Extracellular protons induce a conformational change in the ASIC1 protein independent of the channel pore. Recent studies have found that in acidic conditions, RIP1 binds to the ASIC1a C-terminus, which leads to RIP1 phosphorylation and subsequent cell death through a type of organized necrosis-like process called necroptosis (Wang et al., 2015). This protein-protein association and cell death was detected in affected mouse brain areas in a middle cerebral artery occlusion model of ischemia and was found to be prevented in ASIC1 KO animals (Wang et al., 2015). Necroptosis in neurons was found to be inhibited by intervention of necrostatin-1 (nec1), a RIP1 inhibitor (Wang et al., 2015). Moreover, acid-evoked neuronal death was similarly inhibited by both PcTX and nec1 (Wang et al., 2015). This suggests that inhibiting the activity of RIP1 with necrostatin-1 prevents ASIC-dependent acid-induced death. As RIP1 is not phosphorylated in the presence of PcTX and acid, ASIC1a must be needed for the phosphorylation to occur. Additionally, ASIC1a knockout mouse brains do not exhibit RIP1 phosphorylation following

acidosis in ischemic stroke. This implicates a connection between ASIC1a and RIP1 in this process. Understanding this connection would elucidate the mechanism of ASIC-mediated death and provide a better understanding of interventions targeting acidotoxicity.

Another important aspect of research on this topic is the timing of the mechanism. Studies found that the addition of nec1 in neuronal cultures was only effective at reducing cell death if it was administered before the acid exposure as well as during the exposure (Wang et al., 2015). Additionally, within 30 minutes of acidosis, the amount of RIP1 protein increases but then decreased below its basal levels by 60 minutes (Wang et al., 2015). This timing factor is important to consider in the study of the impact of RIP1 inhibition on ASIC-mediated cell death in our mouse brain slice model. Additionally, RIP1 was found to physically associate with ASIC1a within 30 minutes of ischemia and remains associated for hours (Wang et al., 2015). This supports the importance of the ASIC1-RIP1 interaction in acid-induced neuronal death as acidosis resulting from ischemia lasts for a long time and spreads slowly, leading to delayed ischemic brain injury. This aspect of brain injury is important to study to prevent extensive damage following stroke.

Interestingly, according to recent studies, ASIC current was suggested to be ruled out in the mechanism for ASIC-mediated acid-induced cell death (Wang et al., 2015). With exposure to acidic conditions, homomeric ASIC1a channels become desensitized quickly with low Ca^{2+} permeability (Wang et al., 2015). As most brain acidosis occurs slowly, ASIC-mediated Ca^{2+} influx would be relatively small and not likely to be the cause of major cell death (Wang et al., 2015). Additionally, PcTX and nec1 have been found to inhibit death in neuronal cultures exposed to acidic conditions even without Ca^{2+} present (Wang et al., 2015). Further, desensitized ASIC1a is still able to induce cell death (Wang et al., 2015). Importantly, this cell death may

involve an ASIC1-RIP1 protein interaction independent of ASIC1 current, suggesting that treatments could be developed to target the pathological functions of ASIC1a while leaving critical physiological functions intact.

To study this novel hypothesis on the molecular mechanism of ASIC-mediated cell death and to begin to potentially determine a route through which delta opioid receptor activation is neuroprotective, our *in vitro* slice model described in detail in Chapter 2 can be utilized with an intervention of necrostatin-1, a RIP1 inhibitor. The timing and impact of nec1 action on OGD-induced cell death in this model will help to elucidate the involvement of RIP1 in ASIC-mediated ischemic neuronal death and also explore RIP1 inhibition as a target for the neuroprotective regulation of ASIC-mediated cell death.

Methods

Pre/concurrent Incubation with RIP1 Inhibitor

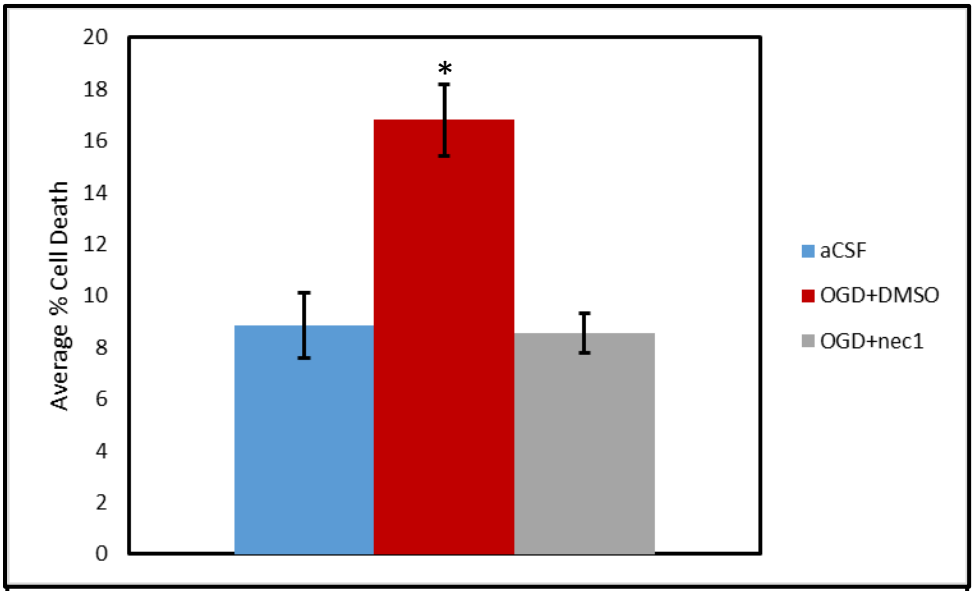
The *in vitro* hippocampal slice model developed and described in Chapter 2 was used following the same protocols for animals, solutions, slicing, recovery, treatments, reperfusion, staining, fixing, imaging, and quantification. The difference in these trials was that an intervention of necrostatin-1 (nec1), a RIP1 inhibitor, or DMSO was added during the last 20 minutes of recovery after slicing as well as during the 20-minute OGD period to allow for pre incubation as well as concurrent incubation with the ischemic event. In addition to these treatments, control slices were exposed to aCSF alone. There were three groups of slice treatments: OGD+nec1 (20 uM), OGD+DMSO (same volume as nec1) and an aCSF control. Slices were treated the same as in the model described in Chapter 2, and cell death was compared between each of the three treatment groups.

Acute (concurrent) Incubation with RIP1 Inhibitor

The same procedure was followed as above, except slices were exposed to nec1 or DMSO only during the OGD period with no pre-incubation.

Results

Comparing average percent cell death between slices exposed to OGD+nec1, OGD+DMSO, and aCSF with the 20 minute pre-incubation period showed a significant difference in cell death levels (n=6, p value=0.00020), with a decrease in cell death in the OGD slices exposed to nec1 to basal levels measured in the aCSF control (**Graph 7**). Average percent cell death observed in the



Graph 7. ASIC WT, OGD+DMSO vs. OGD+Necrostatin-1 vs. Control. Wild type mouse hippocampal brain slices were exposed to 20 minutes pre-incubation of nec1, DMSO, or control, followed by 20 minutes of OGD+DMSO, OGD+nec1, or control (aCSF). Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. One mouse counts as a sample, with n=6. Bars represent hippocampal cell death averaged across the samples (one-way ANOVA p value=0.00020). Bonferroni's Multiple Comparison test showed aCSF vs. OGD+DMSO P <0.001, aCSF vs. OGD+nec1 P >0.05, OGD+DMSO vs. OGD+nec1 P <0.001.

OGD+DMSO group was approximately twice as high as the average percent cell death measured in the control slices as well as in the slices exposed to OGD with necrostatin-1 (**Fig. 8**).

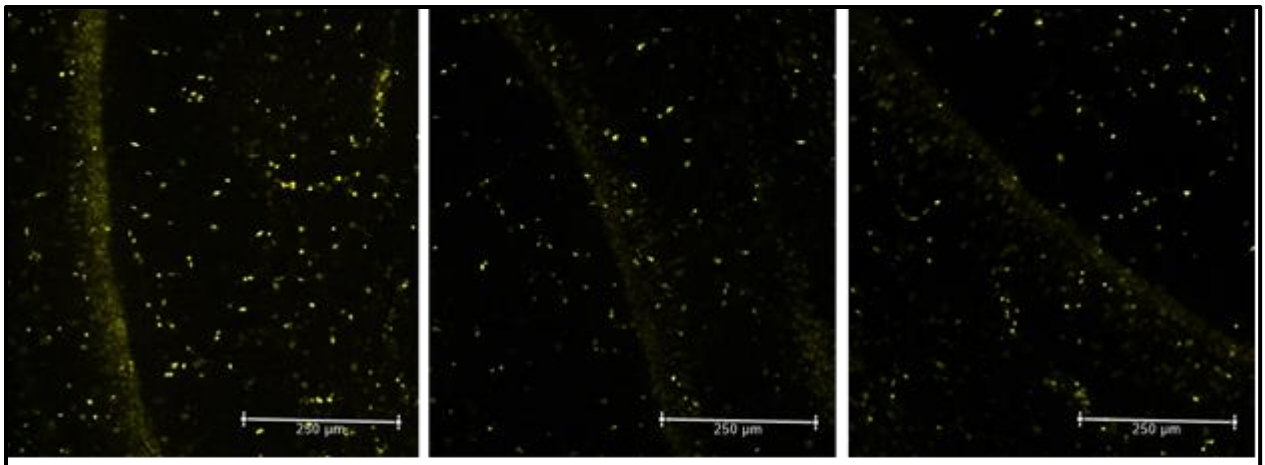
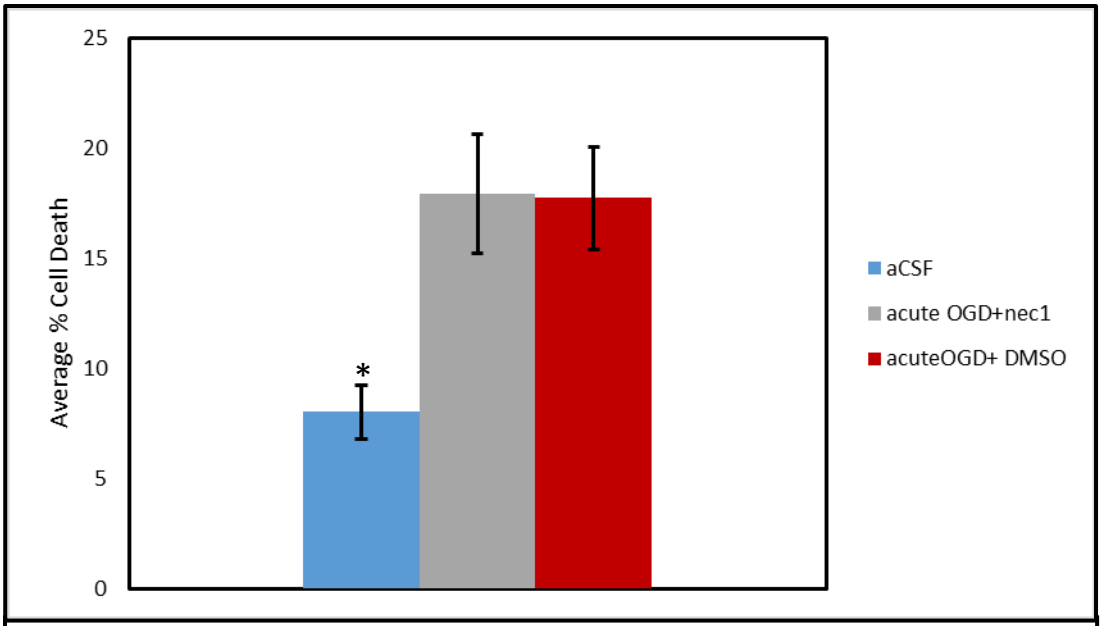


Fig. 8. Imaging: Necrostatin-1. Observing the pyramidal cell layer of these ASIC wildtype hippocampal slices reveals decreased cell death in the OGD-treated slice exposed to necrostatin-1 compared to the OGD-treated slice exposed to DMSO alone. This cell death is decreased approximately to the level of the aCSF control, showing neuroprotection with inhibition of RIP1. Cell death is indicated by yellow fluorescence of cells. Images were taken 25 µm deep in the brain slices.

With acute nec1 administration, or inhibition of RIP1 only during the OGD period, average percent cell death levels were approximately equal between the slices exposed to OGD+nec1 and those exposed to OGD+DMSO, but lower in the aCSF control (**Graph 8**, p value=0.0079). This



Graph 8. ASIC WT, Acute OGD+DMSO vs. Acute OGD+Necrostatin-1 vs. Control. Wild type mouse hippocampal brain slices were acutely exposed to 20 minutes of OGD+DMSO, OGD+nec1, or control (aCSF) with no pre-incubation period. Slices were imaged, and cell death was quantified using FIJI threshold histogram analysis. One mouse counts as a sample, with n=6. Bars represent hippocampal cell death averaged across the samples (one-way ANOVA p value=0.0079). Bonferroni's Multiple Comparison test showed aCSF vs. OGD+nec1 P <0.05, aCSF vs. OGD+DMSO P <0.05, OGD+DMSO vs. OGD+nec1 P >0.05.

shows that necrostatin-1 must be administered prior to the oxygen-glucose deprivation event in order to interfere effectively with this ASIC-mediated cell death pathway.

Conclusion

Reduction of cell death to basal levels in slices exposed to oxygen-glucose deprivation with necrostatin-1 suggests that the observed ASIC-mediated cell death is dependent on RIP1 activity. Involvement of RIP1 in ASIC-mediated cell death induced in this *in vitro* model suggests that RIP1 may be a key player in neuronal death in cerebral acidosis as a result of ischemic stroke. A possible mechanism for neuronal death following ischemic stroke could be an extracellular proton-induced conformational change in the ASIC1a protein leading to RIP1 association at the ASIC1a C-terminus and RIP1 phosphorylation, ultimately leading to necroptosis of the neurons.

The result that ASIC-mediated cell death was not attenuated by inhibition of RIP1 activity during the OGD period alone without necrostatin-1 pre-incubation suggests that inhibiting RIP1 activity with necrostatin-1 is not an effective therapeutic strategy for attenuating brain damage following an ischemic stroke. Evidence from this model indicates that inhibition would have to occur prior to the ischemic event, which is not a practical time frame to prevent brain damage. The knowledge gained from these experiments is useful moving forward in elucidating the mechanism of ASIC-mediated cell death regulation.

Chapter 6: Discussion

First, in this thesis, I developed a mouse hippocampal acute slice model of ischemia to study neuronal death in oxygen-glucose deprivation. Similarly to other models of ischemia

(Bhowmick et al., 2017; Xiong et al., 2004; Yang et al. 2011), the cell death in our acute slice model was shown to be mediated by ASIC1a and was prevented by the specific ASIC1a inhibitor, psalmotoxin-1. Additionally, cell death induced by OGD conditions was reduced relative to the control in the ASIC1 knockout mouse. Second, I used this model to measure the effect of activation of the delta opioid receptor on ASIC-mediated cell death and found attenuation of OGD-induced death. This result replicated our data in neuronal culture and is consistent with previous studies showing attenuation of cell death in ischemia through DOR activation (Chao et al., 2012; Charron et al., 2008; Kao et al., 2008; Oeltgen et al., 2006). A third piece to this study looked to the novel mechanism of this cell death by investigating involvement of the Receptor Interacting Protein Kinase 1 in OGD-induced cell death mediated by ASIC1a. Attenuation of cell death was observed with inhibition of RIP1 activity, a result that is consistent with previous evidence of RIP1 involvement in ASIC-mediated neuronal death (Wang et al., 2015).

The result that RIP1 plays a role in ASIC-mediated cell death in this model has large implications. Studies indicating that ASIC pathological activity is RIP1 dependent show that this ASIC-mediated cell death occurs independent of ASIC current (Wang et al., 2015). Targeting this activity could interfere with ASIC-mediated cell death following an ischemic stroke in a novel, therapeutically meaningful way as cell death inhibition may not interfere with normal ASIC function. Specifically, neuroprotection as a result of activation of the delta opioid receptor may be through the interruption of a part of this RIP1-dependent pathway. This would be consistent with findings in the Askwith lab that DOR activation does not interfere with ASIC current. Importantly, this suggests that potentially RIP1-dependent DOR activation in patients following an ischemic stroke could prove to be a valuable strategy to protect the brain from

permanent injury induced by pathological ASIC activity while leaving important normal ASIC functions intact (**Fig. 9**). The neuroprotection observed in hippocampal oxygen-glucose deprivation with DOR activation in this model suggests that existing DOR agonists, which have already undergone clinical trials for major depressive disorder and anxiety (Hudzik et al., 2011; Pradhan et al., 2011), may prove to be effective in attenuating brain damage following an ischemic stroke in patients.

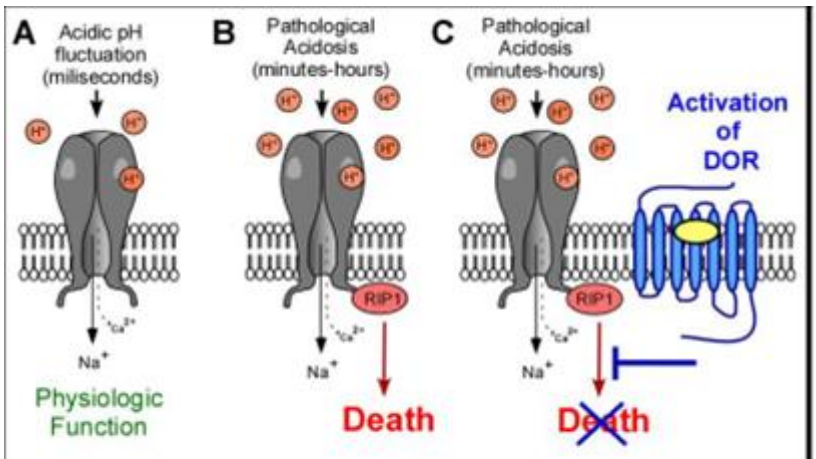


Fig. 9 Prevailing model of ASIC function. A. Under normal conditions, ASIC1a passes sodium to alter synaptic activity in response to extracellular pH changes. **B.** During pathological conditions ASIC1a associates with RIP1 kinase and induces cell death. **C.** Activation of DOR prevents ASIC1a-mediated cell death without impacting ASIC1a current.

This potentially neuroprotective benefit of DOR activation following an ischemic stroke is supported by other studies looking at DOR expression in certain animals. Specifically, animals that undergo hibernation are particularly resistant to decreased oxygen and blood flow during the hibernation period (Johnson and Turner, 2010). Interestingly, studies showed that injecting a DOR agonist intravenously into summer active ground squirrels activated hibernation, and injecting plasma from hibernating woodchucks into mice before inducing focal ischemia was neuroprotective (Johnson and Turner, 2010). Additionally, red-eared slider turtles, which are able to hold their breath for up to 48 hours, exhibit higher levels of DOR expression in the brain than rats, which may contribute to resistance to hypoxia (Johnson and Turner, 2010). Our results are consistent with these observations of DOR-induced resistance to hypoxia and show that DOR activation attenuates ASIC-mediated hippocampal death following oxygen-glucose deprivation in mouse brain slices, a model mimicking cerebral ischemia. Moreover, we have determined that

ASIC-mediated cell death in oxygen-glucose deprivation is dependent on RIP1, which is significant as the mechanism of DOR-induced neuroprotection is not fully understood (Staples et al., 2013). This DOR-induced neuroprotection may be through a novel RIP1-dependent mechanism involving disruption of ASIC1-RIP1 protein associations or RIP1 phosphorylation. Our results lay the framework for further investigation into this mechanism to better understand the exact action of DOR in neuroprotection and to potentially develop new therapeutics for ischemic stroke targeting DOR activation.

Further, the result that DOR activation attenuated neuronal death during the OGD period alone without agonist pre-incubation is therapeutically promising. In contrast, inhibition of RIP1 activity during OGD alone failed to attenuate cell death. One possibility is that DOR may be interfering with a later step in the proposed cell death pathway. However, this effect is likely a result of the longer time period needed for necrostatin-1 to enter the cell and reach its target intracellularly. In contrast, SNC80 reaches its target more quickly to inhibit ASIC-mediated cell death as DOR is a G-protein coupled receptor located in the cell membrane. Therefore, targeting DOR activation may prove to be an effective strategy to inhibit RIP1-dependent ASIC-mediated neuronal death within a clinically relevant time window. Further, it has been shown that inhibiting ASIC1a even hours after blood flow has been restored stops further injury from occurring (Pignataro et al., 2007; Vergo et al., 2011). DOR activation may interfere with RIP1-dependent ASIC-mediated cell death in such a way, representing a clinically viable therapeutic target. More work should be done to look at the specific timing of DOR activation in neuroprotection in the context of ASIC-mediated ischemic cell death as well as the exact mechanism of DOR interference in this RIP1-dependent cell death to further elucidate the mechanism (**Fig. 9**) proposed here.

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